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(54) Title: 5- AND 6-SUCCINIMIDYLCARBOXYLATE ISOMERS OF RHODAMINE DYES

(57) Abstract

Isomerically pure 5- and 6-succinimidylcarboxylates of rhodamine dyes are provided. A key feature in the preparation of the succinimidylcarboxylates is the use of stoichiometric amounts of di-N-succinimidylcarbonate and 4-dimethylaminopyridine for converting the 5- and/or 6-carboxyrhodamines to N-hydroxysuccinimide esters. In chain termination DNA sequencing procedures, using the pure isomeric forms of the activated rhodamines prevents generation of spurious sequence data because of the different electrophoretic mobilities of the isomers.

* See back of page

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5- AND 6-SUCCINIMIDYLCARBOXYLATE ISOMERS OF RHODAMINE DYES

Cross-reference to Related Applications

This is a continuation-in-part of U.S. application Serial Number 06/941,985 filed 15 December 1986, now abandoned.

Field of the Invention

The present invention relates generally to fluorescent probes and/or compounds for labeling molecules of biological or biochemical interest, and more particularly, to N-hydroxysuccinimide activated 5- and 6-carboxyl isomers of rhodamine dyes.

BACKGROUND

Fluorescent labels find widespread applications in molecular biology, microbiology, biochemistry, analytical chemistry, and in the industrial and medical fields rooted in those disciplines, e.g. biotechnology, and environmental, industrial, and diagnostic medicine.

A large number of organic and inorganic fluorescent materials are available. However, many problems are encountered in attempts to construct fluorescent labels out of such materials. The term "fluorescent label" as used herein means an organic molecule having a fluorescent moiety and a linking moiety, such that the linking moiety allows the fluorescent moiety to be covalently attached to a target functionality, e.g. primary amines, secondary amines, or the like.

Some important considerations associated with the construction and use of fluorescent labels include the stability of the bond between the fluorescent moiety

and the linking moiety, the affect that the linking moiety has on fluorescent characteristics before and after attachment to a target functionality, the stability of the bond between the fluorescent moiety and the target functionality, i.e. the strength of the linking moiety after attachment, the stability of the linking functionality to purification procedures, e.g. amide linkages are more stable to hydrolysis than thiourea linkages, the reactivity of the linking functionality with the target functionality, and the like. When multiple fluorescent labels are required, each having distinct fluorescent characteristics, such as nonoverlapping emission bands, selection of labels for a particular application can become very difficult, often necessitating tradeoffs between the desired characteristics of the available fluorescent labels.

When fluorescent labels are used to track target molecules in biochemical separation procedures or in biological processes, two important factors are (1) the extent to which the fluorescent label perturbs the behavior of the target molecules, and (2), if such a perturbation does exist, the uniformity of the perturbation as between target molecules. This latter factor is especially crucial in methods for separating macromolecules on the basis of size by gel electrophoresis. In particular, techniques for sequencing deoxyribonucleic acids (DNAs) rely heavily on the ability to separate oligonucleotides differing by no more than a single base, e.g. Smith et al., Nucleic Acids Research, Vol. 13, pgs. 2399-2412 (1985); Schreier et al., J. Mol. Biol., Vol. 129, pgs. 169-172 (1979); and Sanger et al., J. Mol. Biol., Vol. 143, pgs. 161-178 (1980).

Recently, improvements in such methodologies have made use of multiple fluorescent labels to carry out sequencing automatically on a single columnar gel, e.g.

Smith et al. (cited above), and Smith et al., Nature, Vol. 321, pgs. 674-679 (1986). The choice of fluorescent labels is critical in such technology, and represents a major constraint to expanding the capabilities of the technology. Moreover, in spite of the degree of automation achieved, users still must frequently prepare their own labeled oligonucleotide primers for dideoxy-based sequencing. Such primers must be free of sequence failures associated with the synthesis of the oligonucleotide. A standard technique for oligonucleotide purification involves polyacrylamide gel electrophoresis (e.g. Applied Biosystems Users Bulletin, Issue 13, November 9, 1984, "Evaluation and Purification of Synthetic Oligonucleotides"). If a primer has attached to it dye isomers that have close electrophoretic mobilities, primer purification will be difficult, if not impossible. The use of a single isomer simplifies this type of purification.

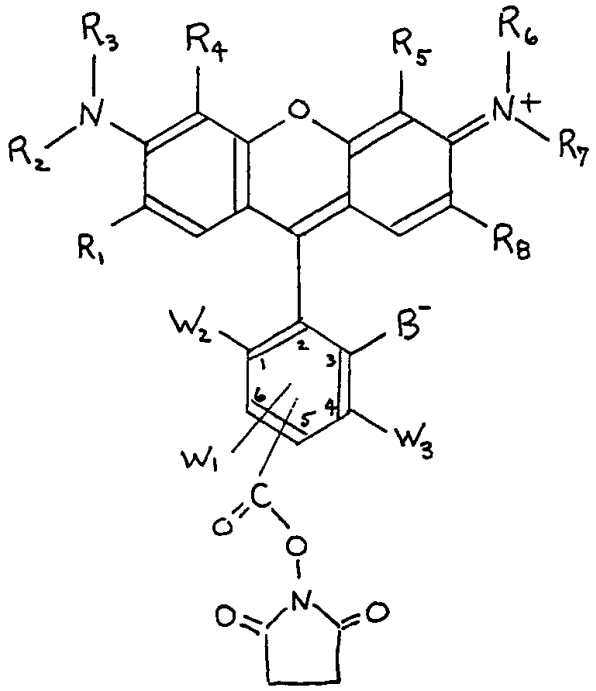
In the electrophoretic separation of fluorescently labeled oligonucleotides important considerations include the relative synthetic yields and stabilities of the fluorescently labeled oligonucleotide conjugates, and the amount of variability in electrophoretic mobility introduced by the attached label. DNA mobility differences characteristic of the different dyes can be corrected with the proper choice of linker used to join the dye to the oligonucleotide; however, if the individual dyes are present as more than one isomer, then the attached label causes significant spreading of bands, or gives rise to multiple bands of identically sized oligonucleotides which, in turn, leads to spurious sequence determination. Unfortunately, rhodamine dyes, an important class of fluorescent labels used in this technology, are available only as mixtures of isomers,

e.g. Haugland, Handbook of Fluorescent Probes and Research Chemicals, (Molecular Probes, Inc. Junction City, Oregon, 1985), and the isomers affect the electrophoretic mobilities of labeled oligonucleotides to different degrees.

In view of the foregoing, the availability of fluorescent labels in pure isomeric forms will increase the applicability of the fluorescent labels, and in particular will increase the sensitivity of fluorescence based methods for distinguishing macromolecules, such as oligonucleotides, separated by gel electrophoresis.

SUMMARY OF THE INVENTION

The present invention includes 5- and 6-succinimidylcarboxylate isomers of symmetric or asymmetric rhodamine dyes, and methods of making and using the same. (Throughout, the Colour Index (Association of Textile Chemists, 2nd. Ed., 1971) numbering scheme is used to identify the carbon atoms of the rhodamine dyes. Carbon atoms in the xanthene-like structure are identified by primed numbers as indicated below, and carbon atoms of the 9'-substituted phenyl are identified by unprimed numbers as indicated below). More particularly, the compounds of the invention are defined by Formula I, and salts thereof, such as carboxylic acid salts, hydrogen halide salts, oxy acid salts, and the like:



Formula I

wherein:

B is an anionic group, preferably carboxylate or sulfonate, and more preferably carboxylate.

R_1 and R_8 taken alone are each hydrogen, halogen, alkyl having from 1 to 8 carbon atoms, alkylether having from 1 to 8 carbon atoms, or alkylthioether having from 1 to 8 carbon atoms, and R_1 taken together with R_2 and R_8 taken together with R_7 are alkyl chains each having from 2 to 5 carbon atoms connecting the 7' carbon to the nitrogen attached to the 6' carbon and connecting the 2' carbon to the nitrogen attached to the 3' carbon, respectively. Preferably, R_1 and R_8 taken alone are each hydrogen, alkyl having from 1 to 3 carbon atoms, chloro, or alkylether having from 1 to 3

carbon atoms, and R_1 taken together with R_2 and R_8 taken together with R_7 each form an alkyl chain having from 2 to 3 carbon atoms connecting the 7' carbon to the nitrogen attached to the 6' carbon and connecting the 2' carbon to the nitrogen attached to 3' carbon, respectively. Most preferably, R_1 and R_8 taken alone are each hydrogen, and R_1 taken together with R_2 and R_8 taken together with R_7 each form an alkyl chain having 3 carbon atoms connecting the 7' carbon to the nitrogen attached to the 6' carbon and connecting the 2' carbon to the nitrogen attached to the 3' carbon, respectively.

R_2 and R_7 taken alone are each hydrogen or alkyl having from 1 to 8 carbon atoms, and R_2 taken together with R_1 and R_7 taken together with R_8 are each alkyl chains having from 2 to 5 carbon atoms as desribed above. Preferably, R_2 and R_7 taken alone are each hydrogen or alkyl having from 1 to 3 carbon atoms, and R_2 taken together with R_1 and R_7 taken together with R_8 are alkyl chains each having from 2 to 3 carbon atoms connecting the 7' carbon to the nitrogen attached to the 6' carbon and connecting the 2' carbon to the nitrogen attached to 3' carbon, respectively. Most preferably, R_2 and R_7 taken alone are hydrogen, methyl or ethyl, and R_2 taken together with R_1 and R_7 taken together with R_8 are alkyl chains each having 3 carbon atoms connecting the 7' carbon to the nitrogen attached to the 6' carbon and connecting the 2' carbon to the nitrogen attached to the 3' carbon, respectively.

R_3 and R_6 taken alone are each hydrogen or alkyl having from 1 to 8 carbon atoms, and R_3 taken together with R_4 and R_6 taken together with R_5 are alkyl chains each having from 2 to 5 carbon atoms connecting the 5' carbon to the nitrogen attached to the 6' carbon and connecting the 4' carbon to the

nitrogen attached to the 3' carbon, respectively. Preferably, R_3 and R_6 taken alone are hydrogen or alkyl each having from 1 to 3 carbon atoms, and R_3 taken together with R_4 and R_6 taken together with R_5 form alkyl chains each having from 2 to 3 carbon atoms connecting the 5' carbon to the nitrogen attached to the 6' carbon and connecting the 4' carbon to the nitrogen attached to the 3' carbon, respectively. Most preferably, R_3 and R_6 taken alone are hydrogen, methyl or ethyl, and R_3 taken together with R_4 and R_6 taken together with R_5 are alkyl chains each having 3 carbon atoms connecting the 5' carbon to the nitrogen attached to the 6' carbon and connecting the 4' carbon to the nitrogen attached to the 3' carbon, respectively.

R_4 and R_5 taken alone are hydrogen, alkyl having from 1 to 8 carbon atoms, halogen, alkylether having from 1 to 8 carbon atoms, or alkylthioether having from 1 to 8 carbon atoms, and R_4 taken together with R_3 and R_5 taken together with R_6 are alkyl chains each having from 2 to 5 carbon atoms as described above. Preferably, R_4 and R_5 taken alone are hydrogen, chloro, alkyl having from 1 to 3 carbon atoms, or alkylether having from 1 to 3 carbon atoms, and R_4 taken together with R_3 and R_5 taken together with R_6 are alkyl chains each having from 2 to 3 carbon atoms as described above. Most preferably, R_4 and R_5 taken alone are hydrogen, and R_4 taken together with R_3 and R_5 taken together with R_6 are alkyl chains each having 3 carbon atoms connecting the 5' carbon to the nitrogen attached to the 6' carbon and connecting the 4' carbon to the nitrogen attached to the 3' carbon, respectively.

W_1, W_2, and W_3 are hydrogen or chloro, and preferably hydrogen.

The invention also includes methods for producing 5- and 6-succinimidylcarboxylates of rhodamine dyes.. One method comprises the steps of (1) separating the 6-

carboxylrhodamine isomer from the 5-carboxylrhodamine isomer, (2) reacting the 6- (or 5-) carboxylrhodamine with equivalent amounts of di-N-succinimidylcarbonate (DSC) and 4-dimethylaminopyridine (DMAP) to form a rhodamine-6- (or -5-) succinimidylcarboxylate in a reaction mixture, and (3) separating the rhodamine-6- (or -5-) succinimidylcarboxylate from the reaction mixture. In another method of the invention a mixture of 5- and 6-succinimidylcarboxylates are formed first, and then separated into isomers. An important feature of the invention is the discovery of a general method for forming N-hydroxysuccinimide (NHS) esters from carboxylic acids of rhodamines by use of stoichiometric amounts of DSC and DMAP.

The invention also includes a method for stabilizing rhodamine carboxylate NHS esters to prevent conversion back to their precursor carboxylic acids. The method comprises treatment of the freshly formed NHS ester with an acidic compound: preferably, a volatile, organic-soluble acid with $pK_a<5$; and more preferably, a volatile, organic-soluble acidic compound with $pK_a<1$, such as HCl or HBr in methanol, or most preferably, trifluoroacetic acid.

As used herein the terms "rhodamine-X-5-succinimidylcarboxylate" and "rhodamine-X-6-succinimidylcarboxlyate" (abbreviated "5-ROX-NHS" and "6-ROX-NHS", respectively) shall refer to the compounds of Formula I wherein R_1 and R_2, R_3 and R_4, R_5 and R_6, and R_7 and R_8 are taken together to form 3 carbon alkyl chains as described above, B is carboxylate, and W_1, W_2, and W_3 are hydrogen, and wherein the succinimidylcarboxylate moiety is attached to the 5- and 6- carbons, respectively. 5-ROX and 6-ROX shall refer to 5- and 6- carboxy precursors of these compounds, respectively. As used herein the terms "tetramethylrhodamine-5-succinimidylcarboxylate" and

"tetramethylrhodamine-6-succinimidylcarboxylate" (abbreviated "5-TMR-NHS" and "6-TMR-NHS", respectively) shall mean the compounds of Formula I wherein R_1, R_4, R_5, R_8, W_1, W_2, and W_3 are hydrogen, B is carboxylate, and R_2, R_3, R_6, and R_7 are methyl, and wherein the succinimidylcarboxylate moiety is attached to the 5- and 6- carbons, respectively. 5-TMR and 6-TMR shall refer to the 5- and 6-carboxy precursors of these compounds, respectively.

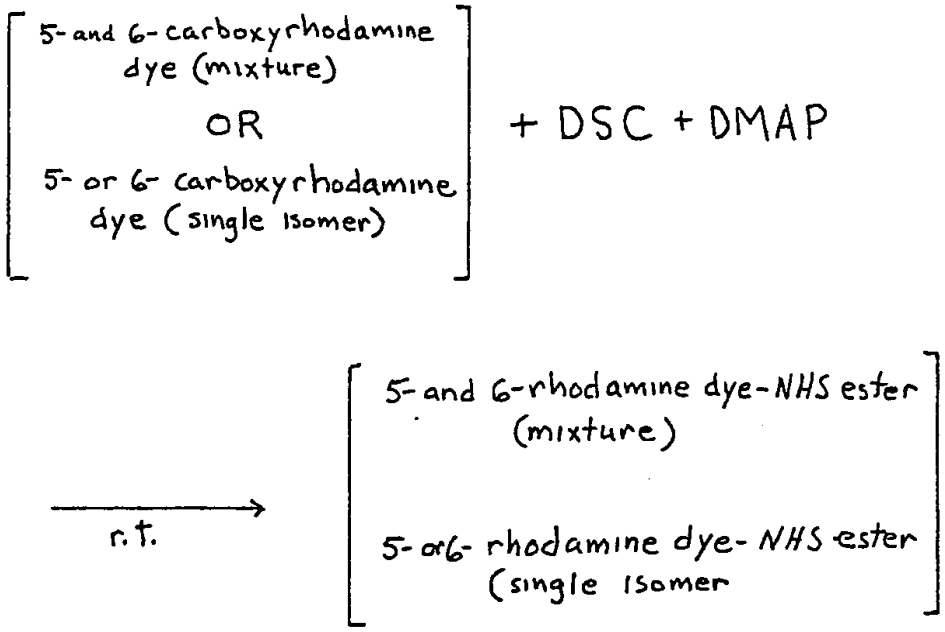
As used herein, the term "rhodamine 110" refers to the compound of Formula I wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, and R_8 are hydrogen, and the term "rhodamine 6G" refers to the compound of Formula I wherein R_1 and R_8 are methyl, R_2 and R_7 are ethyl, and R_3, R_4, R_5, and R_6 are hydrogen.

As used herein in reference to isomeric forms of 5- or 6- carboxylates, or 5- or 6-succinimidylcarboxylates of rhodamine dyes, the term "isomerically pure" means that no detectable double band formation takes place because of the presence of both isomers when an oligonucleotide labeled by a purified isomer of the rhodamine is separated by gel electrophoresis.

DETAILED DESCRIPTION OF THE INVENTION

The invention includes N-hydroxysuccinimide esters of 5- and 6-carboxylrhodamines and methods of making and using the same. Important features of the invention include (1) the reaction condition of having substantially stoichiometric amounts of DSC and DMAP present for esterification of the 5- or 6- forms of the rhodamine dyes to produce high yields of product at room temperature, and (2) the treatment of the freshly synthesized product with an acidic compound, preferably having a pK_a or less than 5, to prevent conversion back

into reactants. The general reaction scheme of the invention is defined by Formula II:



Formula II

The methods comprise reacting the acid form of a 5- or 6-carboxylrhodamine (either as a mixture of isomers, or as pure isomers) with equivalent amounts of di-N-succinimidylcarbonate (DSC) and 4-dimethylaminopyridine (DMAP) in a polar aprotic solvent to form the carboxyl N-hydroxysuccinimide (NHS) ester. Suitable polar aprotic solvents include N,N-dimethylformamide (DMF), pyridine, hexamethylphosphoramide (HMPA), or the like. Most preferably, DMF is used as the reaction solvent. The isomerically mixed NHS esters can be separated into their individual isomers for further use. Most preferably, in order to conserve reagents, the acid forms of the 5- or 6-carboxylrhodamines are first separated into their individual isomers by standard separative techniques, e.g. Edmundson et al., Molecular

Immunology, Vol. 21, pg.561 (1984), and then the individual 5- or 6- carboxyl isomers are reacted as described above to form the 5- or 6-carboxyl NHS esters, respectively, which are separated from the reaction mixture, again using standard techniques.

Some isomeric mixtures of rhodamine dyes for use with the invention are available commercially, e.g. Eastman Kodak Company (Rochester, New York), Molecular Probes, Inc. (Junction City, OR), and others can be synthesized in accordance with the teachings of U.S. Patents 2,242,572; 2,153,059; 3,822,270; 3,932,415; and 4,005,092, all of which are incorporated by reference.

Coumpounds of the invention can be used to label a wide variety of target substances, including proteins, polypeptides, peptides, polysaccharides, polynucleotides, lipids, and combinations and assemblages thereof, such as chromosomes, nuclei, living cells, such as bacteria, other microorganisms, and mammalian cells, tissues, and the like. As used herein the term "polynucleotide" means a single stranded or double stranded chain of DNA or RNA in the size range of about 10-1000 bases in length (if single stranded), or in the size range of about 10-1000 basepairs in length (if double stranded).

Compounds of the invention can be reacted with amino functionalities attached to the 5' or 3' ends of synthetic oligonucleotides and polynucleotides, e.g. as taught by Fung et al, U.S. patent 4,757,141 and Miyoshi et al, U.S. patent 4,605,735.

Compounds of the invention are particularly well suited for identifying classes of polynucleotides that have been subjected to a biochemical separation procedure, such as gel electrophoresis, where a series of bands or spots of target substances having similar physiochemical properties, e.g. size, conformation, charge, hydrophobicity, or the like, are present in a

linear or planar arrangement. As used herein, the term "bands" includes any spacial grouping or aggregation of target substance on the basis of similar or identical physiochemical properties. Usually bands arise in the separation of dye-polynucleotide conjugates by gel electrophoresis.

Classes of polynucleotides can arise in a variety of contexts. For example, they can arise as products of restriction enzyme digests. Preferably, classes identified in accordance with the invention are defined in terms of terminal nucleotides so that a correspondence is established between the four possible terminal bases and the members of a set of spectrally resolvable dyes. Such sets are readily assembled from the dyes of the invention by measuring emission and absorption bandwidths with commercially available spectrophotometers. More preferably, the classes arise in the context of the chemical or chain termination methods of DNA sequencing, and most preferably the classes arise in the context of the chain termination method. In either method dye-polynucleotide conjugates are separated by standard gel electrophoretic procedures, e.g. Rickwood and Hames, Eds., Gel Electrophoresis of Nucleic Acids: A Practical Approach, (IRL Press Limited, London, 1981); or Osterman, Methods of Protein and Nucleic Acid Research, Vol. 1 (Springer-Verlag, Berlin, 1984).

The dye-target substance conjugates are illuminated by standard means for identification, e.g. high intensity mercury vapor lamps, lasers, or the like. Preferably, the dye-target substance conjugates are illuminated by laser light generated by a argon ion laser, particularly the 488 and 514 nm emission lines of an argon ion laser. Several argon ion lasers are available commercially which lase simultaneously at these lines, e.g. Cyonics, Ltd. (Sunnyvale, CA) Model

2001, or the like.

In the chain termination method of DNA sequencing, compounds of the invention can be attached to either primers or dideoxynucleotides. For example, they can be linked to an amino functionality on the 5' end of the primer, e.g following the teaching in Fung et al, U.S. patent 4,757,141 which is incorporated herein by reference; on the base of a primer; or on the base of a dideoxynucleotide, e.g. via the alkynylamino linking groups disclosed by Hobbs et al, European patent application number 87305844.0 which is incorporated herein by reference.

EXAMPLES

The following examples serve to illustrate the present invention. The concentrations of reagents, temperatures, and the values of other variable parameters are only to exemplify the invention and are not to be considered limitations thereof.

EXAMPLE 1. 6-TMR-NHS

6-TMR acid was separated from a mixture of the 5- and 6-TMR acid isomers by column chromatography. 8.82 mg of 6-TMR acid and 10.5 mg of DSC were dissolved in 0.5 ml of dry DMF under argon. 0.09 ml of a 0.5 molar solution of DMAP in tetrahydrofuran (THF) was added in one portion. After 2 hours at room temperature, the mixture was taken into 50 ml of chloroform and washed three times with a 1:1 solution of brine:water. The chloroform was evaporated and the residue was purified on a 20 g silica gel column (300:30:8 methylene chloride:methanol:acetic acid elution). Fractions with R_f of about 0.4 were evaporated to dryness, yielding 8.6 mg of 6-TMR-NHS as its acetic acid salt.

EXAMPLE 2. 5-TMR-NHS

5-TMR-NHS was prepared from 82.3 mg of 5-TMR acid, 75 mg of DSC, 0.70 ml of 0.5 molar DMAP in THF in 2 ml dry DMF, as described in Example 1.

EXAMPLE 3. 6-ROX-NHS

6-ROX acid was separated from a mixture of 5- and 6- acid isomers by column chromatography. 46.2 mg of 6-ROX acid and 58 mg of DSC were dissolved in 2 ml of dry DMF under argon and 0.45 ml of a 0.5 molar solution of DMAP in THF was added in one portion. After 1.5 hours at room temperature, the mixture was taken into 100 ml chloroform and washed four times with a 1:1 solution of brine:water. The chloroform was evaporated and the residue was purified on a 40 g silica gel column (300:30:8 methylene chloride:methanol:acetic acid elution). Fractions with R_f of about 0.5 were evaporated to dryness, yielding 56.4 mg of 6-ROX-NHS as its acetic acid salt.

EXAMPLE 4. 5-ROX-NHS

5-ROX-NHS was prepared from 27.4 mg of 5-ROX acid, 30.2 mg of DSC, 0.24 ml of 0.5 molar DMAP in THF in 1.0 ml dry DMF, as described in Example 3.

EXAMPLE 5. 6-TMR Derivatized Aminoethyloligonucleotides

0.50 mg of 6-TMR-NHS was added to a solution consisting of 20 microliters of 1.0 millimolar 5'-aminoethylphosphate oligonucleotide (an 18-mer) in water and 10 microliters of 1 molar $NaHCO_3/Na_2CO_3$ buffer, pH 9.0. After 1 hour in the dark, the solution was passed through a 10 ml Sephadex G-25 (medium) column with 0.1 molar triethylammonium acetate buffer, pH 7.0. The band of colored material eluting in the exclusion volume was collected. Reverse phase

HPLC showed greater than 90% conversion of the oligonucleotide to the fluorescent product.

EXAMPLE 6. 5-TMR Derivatized Aminoethyloligonucleotide

5-TMR-NHS was reacted with a 5'-aminoethylphosphate oligonucleotide (18-mer) as described in Example 5. The labeled oligonucleotide was removed from the reaction mixture as described in Example 5. The products from this example and from Example 5 were purified by preparative HPLC, 3' end-labeled with [alpha-^{32}P]-cordycepin-5'-triphosphate via terminal deoxynucleotidyltransferase, and electrophoresed side by side on a 20% polyacrylamide gel. Exposure of the gel onto x-ray film showed that the product from Example 5 moved the equivalent of about 1/2 nucleotide slower on the gel than the product from the present example.

EXAMPLE 7. 6-ROX Derivatized Aminoethyloligonucleotide

To a solution consisting of 20 microliters of 1.0 millimolar 5'-aminoethylphosphate oligonucleotide (18-mer) in water and 10 microliters of 1 molar $NaHCO_3/Na_2CO_3$ buffer, pH 9.0, was added 0.42 mg 6-ROX-NHS, followed by 5 microliters of DMF. After 1 hour in the dark, the solution was passed through a 10 ml Sephadex G-25 (medium) column with 0.1 molar triethylammonium acetate buffer, pH 7.0. The band of colored material eluting in the exclusion volume was collected. Reverse phase HPLC showed greater than 70% conversion of the oligonucleotide to the fluorescent product.

EXAMPLE 8. 5-ROX Derivatized Aminoethyloligonucleotides

5-ROX-NHS was reacted with a 5'-aminoethylphosphate oligonucleotide (18-mer) as described in Example 7. The labeled oligonucleotide was removed from the reaction mixture as described in Example 7. The products from the present example and from Example 7 were purified by preparative HPLC, 3'-end labeled as in Example 6, and electrophoresed side by side on a 20% polyacrylamide gel. Exposure of the gel onto x-ray film showed that the product from Example 7 moved the equivalent of about 1/2 nucleotide slower than the product of the present example.

EXAMPLE 9. A Stable Formulation of Rhodamine NHS esters

a) 0.44 mg of 6-carboxy-X-rhodamine NHS ester from Example 3 and 80 ul of 0.01 molar ethanol amine in methanol were combined. Reverse phase HPLC of the reaction mixture with acetonitrile and 0.1 molar triethylammonium acetate buffer (pH=7.0) showed that the product was composed of 70% X-rhodamine acid and 30% of X-rhodamine NHS ester (observed as the ethanolamide of 6-carboxy-X-rhodamine from its reaction with ethanol amine).

b) 0.15 g of 6-carboxy-X-rhodamine NHS ester from Example 3 were dissolved in 100 m. of chloroform; the chloroform solution was washed two times with 0.5 molar sodium bicarbonate, dried with sodium sulfate, filtered, treated with 0.1 ml of acetic acid and evaporated to dryness. 0.35 mg of the product was treated exactly as in a); reverse phase HPLC showed 20% 6-carboxy-X-rhodamine acid and 80% of 6-carboxy-X-rhodamine NHS ester.

c) 0.15 g of 6-carboxy-X-rhodamine NHS ester from Example 3 was treated exactly as in b), except that trifluoroacetic acid was substituted for acetic acid.

0.19 mg of the resulting solid were treated exactly as in a); reverse phase HPLC showed <5% 6-carboxy-X-rhodamine acid and >95% 6-carboxy-X-rhodamine NHS ester.

EXAMPLE 10. Use of 6-succinimidylcarboxylate Isomers in DNA Sequencing by the Dideoxy Chain Termination Method

DNA sequence analysis is highly useful, both scientifically and commercially. The two primary techniques for sequencing DNA fragments are chemical methods, e.g., Maxam and Gilbert, Proc. Nat. Acad. Sci., Vol. 74, p. 560 (1970), and dideoxy chain termination methods, e.g., Smith, Methods in Enzymology, Vol. 65, Grossman and Moldave, eds., pgs. 560-580 (Academic Press, New York, 1980), and Sanger et al., Proc. Natl. Acad. Sci., Vol. 74, pgs. 5363-5367 (1977). The method of the invention can be applied with either technique to substitute fluorescent labels for radioactive labels. In this example, it is shown how the subject invention is used in the dideoxy chain termination method of DNA sequencing.

In the dideoxy chain termination method a DNA strand to be sequenced is used as a template for Escherichia coli DNA polymerase I. A primer is annealed to a piece of single-stranded DNA containing the template, and then it is extended enzymatically up to 400 or more nucleotides in the presence of radioactively labeled deoxyribonucleoside triphosphates, e.g. ^{32}P-labeled adenosine triphosphate, and the dideoxyribonucleoside triphosphate analog of one of the four nucleotides. That is, four separate reactions are carried out each including a different dideoxy analog. Because DNA chain growth requires the addition of deoxyribonucleotides to the 3'-hydroxyl, incorporation of a dideoxyribonucleotide terminates

chain growth. Incorporation of the dideoxy analog in place of the normal nucleotide occurs randomly, so that each of the four reactions generates a heterogeneous population of labeled strands terminating with the same nucleotide, which can be separated electrophoretically according to chain length. That is, four classes of oligonucleotides are established based on the type of terminal dideoxyribonucleoside which is present. A single stranded DNA phage M13 is used to clone copies of the DNA fragment to be sequenced. When a sufficient quantity of M13 is cloned, the M13 DNA is purified and separated into four aliquots. In each aliquot the synthesis or chain growth reaction takes place in the presence of the respective dideoxyribonucleotides.

In accordance with the invention, instead of labeling oligonucleotides by incorporation of radioactive nucleotides during the chain growth phase, primers are synthesized and then labeled by attaching an appropriate linking functionality and reacting it with a dye. An amine linking functionality is attached by reacting the primers with 2-methoxy-3-trifluoroacetyl-1,3,2-oxazaphosphacyclopentane, disclosed in U.S. patent 4,757,141 (mentioned above), or other phosphoramidite linking agents, e.g. as disclosed by Smith et al. Nucleic Acids Research, (cited above). An important constraint on this variation of the dideoxy chain termination method is that the fluorescent dyes be spectrally resolvable. As used herein the term "spectrally resolvable" means that the fluorescent emission bands of the four dyes are sufficiently non-overlapping so that they can be distinguished using standard photodetection methods. Another important constraint is that the dyes must be electrophoretically compatible. As used herein the term "electrophoretically compatible" means that the different dyes must not alter the electrophoretic

mobilities of the labeled polynucleotides so that relative band position on a gel is directly related to relative size of the labeled polynucleotide.

Protecting groups are removed and 6-ROX-NHS reacted with the deprotected 5'-amine as described in Example 7 to form ROX-primer conjugates. In a separate synthesis, protecting groups are removed and 6-TMR-NHS is reacted with the deprotected 5'-amine as described in Example 5 to form TMR-primer conjugates. The primer conjugates are then used in accordance with the dideoxy chain termination method as disclosed by Smith et al. Nucleic Acids Research and Nature (both cited above and both incorporated by reference). Preferably, carboxyfluorescein and 2',7'-dimethoxy-4',5'-dichlorocarboxyfluorescein, attached to the 5'-amines of their respective dideoxy-oligonucleotides via hexyl linkages, are used whenever 6-ROX and 6-TMR are attached via ethyl linkages.

The relative size of the extended primers and the nature of their terminal dideoxyribonucleotides are determined as bands of homogeneously sized extended primers travel down an electrophoresis lane and are detected by a fluorimeter or spectrophotometer after illumination. Preferably, for the above selection of dyes, bands are illuminated with both 514 nm and 488 nm laser light, either sequentially or simultaneously. Homogeneously sized extended primers labeled with either 6-ROX or 6-TMR travel down the electrophoresis gel as narrow bands, while homogeneously sized extended primers labeled with mixtures of 5-ROX and 6-ROX, or 5-TMR and 6-TMR travel down the gel as two bands giving incorrect sequence information.

The foregoing disclosure of preferred embodiments of the invention has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise

form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application, to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

I CLAIM:

1. A compound comprising isomerically pure 5-succinimidylcarboxylrhodamine, or salts thereof.

2. The compound of claim 1 wherein said 5-succinimidylcarboxylrhodamine is rhodamine-X-5-succinimidylcarboxylate, rhodamine-110-5-succinimidylcarboxylate, rhodamine-6G-5-succinimidylcarboxylate, or tetramethylrhodamine-5-succinimidylcarboxylate.

3. A compound comprising isomerically pure 6-succinimidylcarboxylrhodamine, or salts thereof.

4. The compound of claim 3 wherein said 6-succinimidylcarboxylrhodamine is rhodamine-X-6-succinimidylcarboxylate, rhodamine-110-6-succinimidylcarboxylate, rhodamine-6G-6-succinimidylcarboxylate, or tetramethylrhodamine-6-succinimidylcarboxylate.

5. A method of forming an N-hydroxysuccinimide ester of a rhodamine carboxylic acid comprising the step of:

reacting the rhodamine carboxylic acid with equivalent amounts of di-N-succinimidylcarbonate and 4-dimethylaminopyridine to form a succinimidylcarboxylate.

6. The method of claim 5 further including the step of treating said succinimidylcarboxylate with a volatile acidic compound having a pK_a of less than 5.

7. The method of claim 6 wherein said volatile acidic compound has a pK_a of less than 1.

8. The method of claim 7 wherein said rhodamine carboxylic acid is selected from a group consisting of isomerically pure 5-carboxylate rhodamine, isomerically pure 6-carboxylate rhodamine, and a mixture of 5- and 6-carboxylate rhodamine.

9. The method of claim 8 wherein said volatile acidic compound is trifluoroacetic acid.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05078

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07D 455/04; C07D 405/12

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	546/37, 548/525

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with Indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 4,213,904 (HAUGLAND) 22 July 1980 Note: Column 1, line 48 to column 2, line 7.	1-9
X	US, A, 4,745,181 (LAW ET AL) 17 May 1988 Note: Column 1, line 37 to column 2, line 20.	1-4
Y	Tetrahedron Letters, Vol 23, No. 17, pp. 1739-1742 (1982); Brunelle, Note: pages 1739-1742.	5-9
Y	Tetrahedron Letters, No 49, pp. 4745-4746. (1979); Ogura et al. Note: pages 4745-4746.	5-9

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
18 DECEMBER 1990	22 JAN 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	for Amanda Robinson JOSEPH P. BRUST

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